Repair of Heat-Injured *Staphylococcus aureus* 196 E on Food Substrates and Additives and at Different Temperatures

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ABSTRACT

Repair of heat-injured *Staphylococcus aureus* 196E was studied on a newly developed agar medium containing 25% ground beef. The cells were heat-injured at 50 C in 0.1 M potassium phosphate buffer (pH 7.2). After being heated, the cells were surface plated on: Tryptic soy agar (TSA); TSA + 7% NaCl (TSAS); ground beef agar (GBA) with and without various additions; and meat/food agar. Repair is defined as the number of organisms growing on GBA, GBA + addition, or meat/food agar that is greater than the number growing on TSAS by at least one log cycle. The following additives incorporated into GBA permitted repair of heat-injured *S. aureus*: nitrite (up to 400 ppm), ascorbate (up to 500 ppm), lactic acid (down to pH 5.5), liquid smoke preparations, and water activity-lowering substances including glycerol (10%), NaCl (2.5%), KCl (5%) and sucrose (30%). Cells regained salt tolerance on TSA when incubated at temperatures from 20 to 45 C, but not at 16 or 50 C. Repair was most rapid at 35 C. When ground beef was replaced in the plating medium, repair occurred on frankfurter and chili beef soup agars, but not on pepperoni and Lebanon bologna agars. Repair of heat-injured *S. aureus* can take place on meat-foods, in the presence of various meat additives, and at temperatures from 20 to 45 C.

Staphylococcus aureus is often associated with both fresh and cured meat products (*6,8*). In addition, fresh and processed meats can support both growth and toxin production by *S. aureus* (*6,8,11*). In 1977, red meat, poultry and processed meats were responsible for 68% (17/25) of the outbreaks of food poisoning by *S. aureus* (*2*) with ham alone responsible for 36% (9/25) of these outbreaks.

It is known that food processing operations, such as drying, freezing and heating, will injure or stress microorganisms including *S. aureus* (*1*). This injury is manifested by the inability of the cells to form colonies in the presence of selective agents such as salt or dyes which non-injured cells can tolerate. Repair, i.e., regaining the ability to form colonies in the presence of the selective agent, has been studied in culture media, and the specific nutrient requirements have been determined. Amino acids, glucose, phosphate and Mg^{+2} are required for repair of *S. aureus* (*3,4*). There have been no studies on the ability of injured cells to repair in food products or on the influence of various food additives (except NaCl). In this study, we describe a meat/food agar plating system which permitted us to evaluate the ability of heat-injured *S. aureus* to repair on meat products, in the presence of meat additives and under temperature conditions found in storage of meat products.

MATERIALS AND METHODS

Culture and growth conditions

Staphylococcus aureus 196E was used throughout these studies. It was grown in Tryptic soy broth (TSB; Difco; 100 ml TSB in a 1-L Erlenmeyer flask) incubated for 16 h at 35 C and 200 rpm.

Cell suspension

Following incubation, the contents of the shake culture were centrifuged at 16,300 × *g* (2-4 C), and washed 3× with 0.1 M potassium phosphate buffer (pH 7.2) and resuspended in 5 ml of buffer.

Heat injury

The cell suspension was added to 200 ml of 0.1 M potassium phosphate buffer equilibrated to 50 C. Heat injury was accomplished by exposing the cell suspension at 50 C with agitation. At intervals, 5 ml portions of the heated suspension were removed, placed in cold sterile tubes, cooled immediately in an ice slush bath and surface plated on Tryptic soy agar (TSA, Difco) which permits growth of both injured and non-injured cells, and on TSA + 7% NaCl (TSAS) on which only non-injured cells can grow (*4*).

Ground beef/meat substrate agar

For investigations of the ability of various meat substrates and additives to support repair of heat-injured *S. aureus*, the suspension of heated cells was plated on a ground beef agar (GBA) with and without various additions, or on meat-food agar substrates. The ground beef/meat-food agar substrate was prepared as follows: 50 g of ground beef or meat/food were sterilized by autoclaving, added to a sterile 500-ml Waring blendor jar, 50 ml sterile distilled water added, and the mixture blended to a smooth slurry (ca. 3 min). After blending, the slurry was added to 100 ml of a 3% agar (Difco) solution, swirled to achieve a uniform distribution, then poured into sterile Petri dishes and allowed to solidify. Additives, such as salt and sugar, were added to the 3% agar solution and sterilized by autoclaving. Heat labile substances were dissolved in a minimal amount of distilled water, filter

sterilized (Millipore, 0.45 -mμ filter) and added to the mixture just before pouring.

Except for the temperature study, a food agar supported repair of heat-injured *S. aureus* if the following two criteria were met: (a) if the non-heated cells formed colonies on it and gave counts similar to TSA and (b) if the count on the food agar was at least one log cycle above the TSAS count. Though criterion (b) represents only a relatively small amount of repair (quantitatively), one log cycle is generally accepted as the error of the plating procedure. Thus, if all *S. aureus* in a food were heat-injured and if both criteria (a) and (b) were met, *S. aureus* could repair and grow in the food and the food become hazardous. Implicit in this definition is the notion, accepted a priori, that injured cells must repair before they will form colonies even on TSA.

Temperature

Except for the experiment in which repair at different temperatures was studied, all plates were incubated at 35 C. For studies of the influence of temperature on repair of heat-injured *S. aureus*, several duplicate plates of TSA were inoculated with heated cells and incubated at temperatures from 9 to 50 C. All samples were compared to the heated culture incubated at 35 C; this was the zero time of repair value plated on TSAS. After various time intervals at the different temperatures, the TSA plates were overlaid with a large excess of mannitol salt agar (MSA), and then incubated at 35 C. An increase in the number of salt-tolerant, mannitol-positive colonies compared to TSAS indicated that repair had taken place at that temperature. Repair is therefore defined as log MSA overlay plate count at the various temperatures minus log TSAS count at zero time of repair.

pH

The pH of the various agars was determined by touching their surfaces with a combination surface electrode (Owens-Illinois[1], pH 2000) connected to a Beckman pH meter (Model 76).

RESULTS AND DISCUSSION

Effect of additives on repair

Since many additives used in meats as well as the sausage fermentation process lower the pH of the product, the effect of different levels of lactic acid (and different pH values) on the repair of heat-injured *S. aureus* was first investigated (Fig. 1). Repair occurred in the presence of 10 mM lactic acid (pH 5.5); however, no repair occurred in 15 or 20 mM lactic acid (pH 5.1). It is interesting to note that there were fewer cells on GBA containing 20 mM lactic acid than on 15 mM lactic acid even though their pH values were the same. The lactic acid concentrations encountered in most meat products, except for fermented sausages and pickled products, should permit repair of heat-injured *S. aureus*. Iandolo and Ordal (*4*), working with *S. aureus* MF31, observed virtually no repair at pH 5.0 and below in TSB during a 2-h repair period and thus agree with our finding that pH values near 5.0 appear to be limiting for repair.

Various additives permitted in meat products were incorporated into GBA to determine if they would support repair of heat-injured *S. aureus* (Fig. 2 and 3). When nitrite was added to the GBA, sufficient lactic acid also was added to lower the pH of the medium to below 6. This extra addition was used since nitrite is an effective

[1]*Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.*

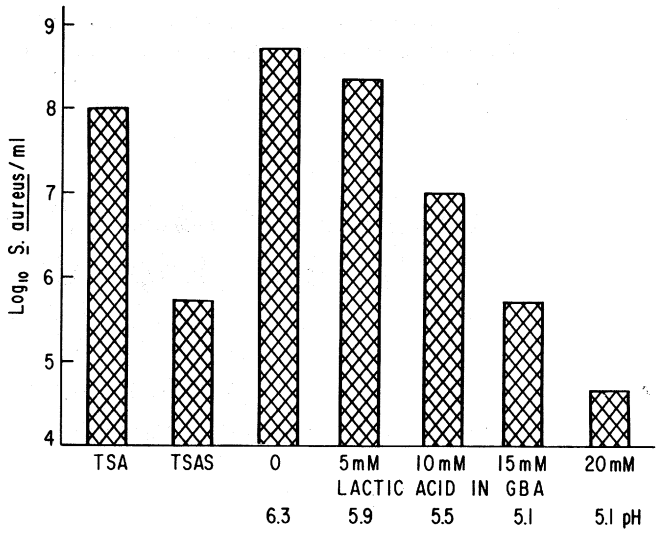

Figure 1. *Influence of lactic acid in ground beef agar (GBA) on repair of* S. aureus *196E heated 90 min.*

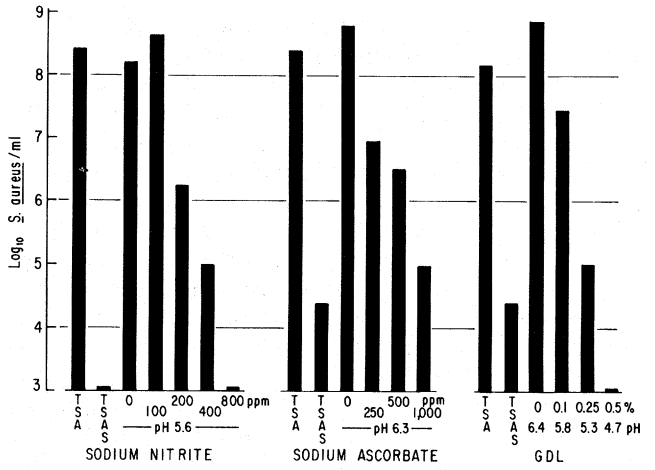

Figure 2. *Influence of sodium nitrite, sodium ascorbate and glucono-delta-lactone (GDL) in GBA on repair of* S. aureus *196E heated 90 min.*

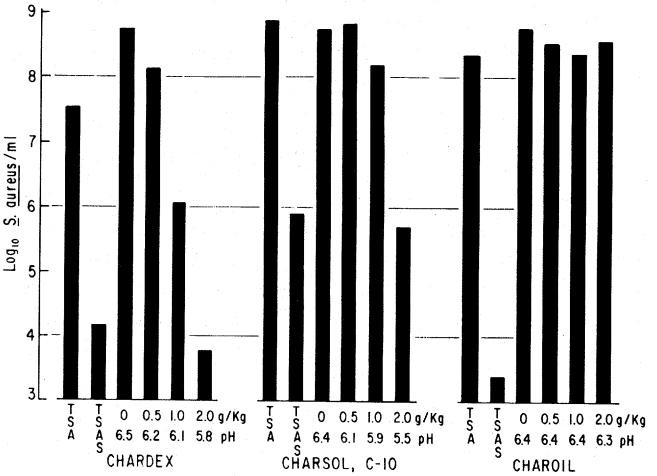

Figure 3. *Influence of liquid smoke preparations[1] in GBA on repair of* S. aureus *196E heated 90 min. ([1]Red Arrow Products Company, Manitowoc, WI. Two grams was highest level tested. This was the highest level recommended by the manufacturer and the level that is permitted to be added; 2-g level is equivalent to 8 oz/100 lb meat).*

antibacterial agent only at pH values below 6. Nitrite allowed repair at levels up to 400 ppm. Sodium ascorbate allowed repair to occur at levels up to 500 ppm. Because erratic and irreproducible results were obtained, no data on nitrite-ascorbate combinations are given even though these were tested. Glucono-delta-lactone at 0.5% (maximum level permitted in sausages) inhibited repair, probably because of the low pH (4.7). The two liquid smoke preparations, which did not permit repair at the 2-g level, have higher acid contents (lower pH values, Fig. 3) than the one which permitted repair at the level); all three have similar amounts of phenolics (company literature, Red Arrow Products Company, Manitowoc, WI).

When low levels of water activity (a_w)-lowering substances were added to GBA, *S. aureus* could repair the heat injury in their presence (Fig. 4). A_W values obtained from Scott (*9*) for ideal solutions of NaCl, sucrose and glycerol for the levels of these substances which supported repair of cells heated for 90 min are all about 0.98. These data indicate that above an a_w of 0.98, a_w has little effect on repair of heat injury in *S. aureus*. Because of the way in which these studies were performed, the exact level that did not permit repair could not be determined for the various a_w-lowering substances. However, the a_w levels commonly found in meat products would permit repair.

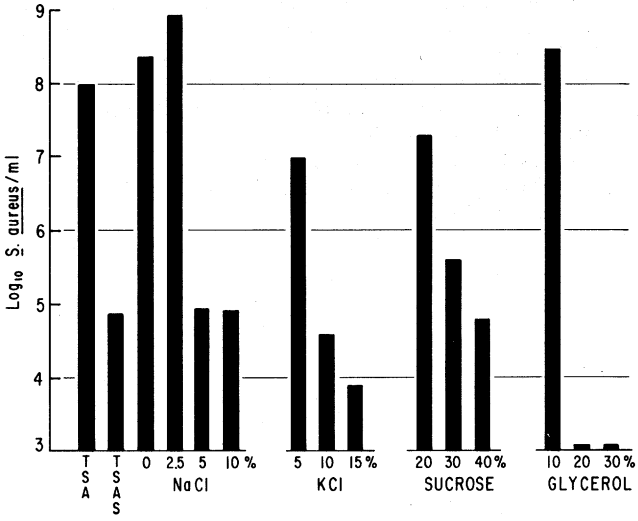

Figure 4. *Influence of water activity-lowering substances in GBA on repair of* S. aureus *196E heated 90 min.*

Meat food substrates

The ability of four individual meat/foods to permit repair was investigated by substituting them for the ground beef in GBA (Fig. 5). Data in Fig. 1 regarding the influence of lactic acid explains the lack of repair in pepperoni agar (pH 4.9). Lebanon bologna agar (pH 4.8) contained inhibitory factors other than lactic acid since even unheated cells could not form colonies. Only very limited data on repair of injured cells, especially *S. aureus* in food products, are available. Stiles and Witter (*10*) reported that heat-injured *S. aureus* would repair in milk. Wilson and Davis (*12*) observed repair of

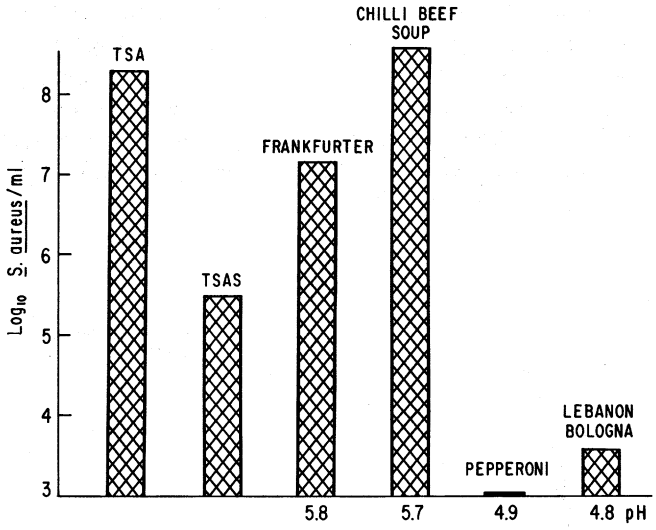

Figure 5. *Influence of meat food agar substrates on repair of* S. aureus *196E heated 90 min.*

heat-injured *Salmonella senftenberg* in a broth system containing materials (at the 10% level) such as bone meal, whole egg and fresh egg albumen. Janssen and Busta (*5*) found that repair of freeze-injured *Salmonella anatum* was promoted by milk components. With freeze-injured *Escherichia coli*, Ray and Speck (*7*) observed some repair in egg and crab meat broth. Our data (Fig. 1-5) indicate that many meat products would permit repair of heat-injured *S. aureus* and that additives at levels encountered in meat products would not inhibit that repair.

Temperature

Heat-injured *S. aureus* can regain salt tolerance on TSA incubated at temperatures from 16 to 45 C, with the most rapid recovery occurring at 35 C and only limited recovery at 16 C (Fig. 6). Iandolo and Ordal (*4*), using a 2-h repair period in TSB, reported that 20 to 37 C were the best recovery temperatures, with very little recovery at 45 C. Their limited recovery at 45 C is in contrast to our finding that 45 C was almost as good as 35 C. Possible explanations for this may be strain differences, the use of TSB vs. TSA, and repair in broth vs. repair on solid media. These data on the effect of temperature on repair suggest that food receiving only a relatively mild heat treatment should be refrigerated to 16 C or below or held at 50 C or above to prevent repair of heat-injured *S. aureus*.

In addition to the basic finding that heat-injured *S. aureus* will repair on GBA, GBA plus additives found in many meat products, meat/food agars and at various temperatures, we also observed degrees of heat injury. Two illustrations of this phenomenon are shown in Fig. 7a and b. In Fig. 7a, cells heated for different times were plated on GBA containing increasing levels of glycerol. Though the number of injured cells (difference between TSA and TSAS) did not change from 60 to 90 min, the cells heated for longer times were more sensitive to glycerol. In Fig. 7b, it can be seen that cells heated for longer times required longer times at 16 C to regain salt

tolerance. This effect of temperature on repair was also seen at 20 and 35 C. The overall effect of degrees of injury was observed with intermediate levels of nitrite, lactic acid and sodium ascorbate as well as the 1-g level of Chardex. Thus, extended heating seems to make the cells more sensitive to various agents while not increasing the actual number of injured cells.

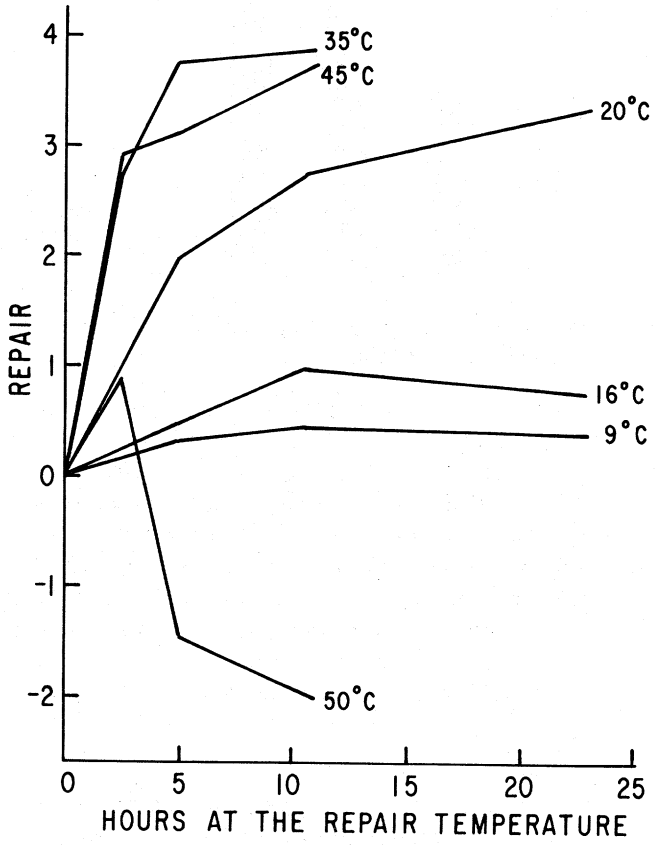

Figure 6. *Influence of temperature of repair on repair of* S. aureus *196E heated 90 min. Repair equals log MSA overlay plate count at the different temperatures minus log TSAS count at zero time.*

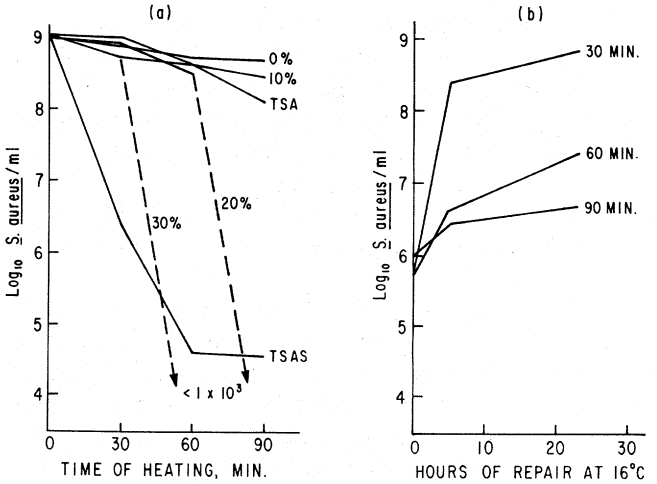

Figure 7. *Influence of length of heating on repair of* S. aureus *196E: (a) effect of different levels of glycerol in GBA on repair of cells heated different lengths of time and (b) effect of heating time (30, 60 or 90 min) on repair at 16 C. (Repair is indicated by an increase in number of salt-tolerant, mannitol-positive colonies on MSA overlay plates.)*

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REFERENCES

1. Busta, F. F. 1976. Practical implications of injured microorganisms in food. J. Milk Food Technol. 39:138-145.
2. Center for Disease Control. 1979. Foodborne and Waterborne Disease Outbreaks Annual Summary 1977. Issued Aug. 1979.
3. Hughes, A., and A. Hurst. 1976. Magnesium requirement of *Staphylococcus aureus* for repair from sublethal heat injury. Can J. Microbiol. 22:1202-1205.
4. Iandolo, J. J., and Z. J. Ordal. 1966. Repair of thermal injury of *Staphylococcus aureus*. J. Bacteriol. 91:134-142.
5. Janssen, D. W., and F. F. Busta. 1973. Influence of milk components on the injury, repair of injury, and death of *Salmonella anatum* cells subjected to freezing and thawing. Appl. Microbiol. 26:725-732.
6. Minor, T. E., and E. H. Marth. 1972. *Staphylococcus aureus* and staphylococcal food intoxications. IV. Staphylococci in meat, bakery products, and other foods. J. Milk Food Technol. 35:228-241.
7. Ray, B., and M. L. Speck. 1973. Enumeration of *Escherichia coli* in frozen samples after recovery from injury. Appl. Microbiol. 25:499-503.
8. Riemann, H., W. H. Lee, and C. Genigeorgis. 1972. Control of *Clostridium botulinum* and *Staphylococcus aureus* in semi-preserved meat products. J. Milk Food Technol. 35:514-523.
9. Scott, W. J. 1957. Water relations of food spoilage microorganisms. Adv. Food Res. 7:83-127.
10. Stiles, M. E., and L. D. Witter. 1965. Thermal inactivation, heat injury, and recovery of *Staphylococcus aureus*. J. Dairy Sci. 48:677-681.
11. Tatini, S. R. 1973. Influence of food environments on growth of *Staphylococcus aureus* and production of various enterotoxins. J. Milk Food Technol. 36:559-563.
12. Wilson, J. M., and R. Davis. 1976. Minimal medium recovery of thermally injured *Salmonella senftenberg* 4969. J. Appl. Bacteriol. 40:365-374.